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GC-MS analysis of the alkaloid extract of *Epdedra equisetina* and *in silico* anti-inflammatory activity of its alkaloids

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Epdedra equisetina is a potential medicinal plant in Tajikistan. It has been used for a long time in Tajik folk medicine for colds, joint pain, rheumatism, malaria, stomach and liver disorders. The alkaloid extract of *Epdedra equisetina* is obtained by acid-base extraction and analyzed by gas chromatography-mass spectrometry (GC-MS) and column chromatography (CC). The alkaloid extraction yield of *Ephedra equisetina* is 1.57%. Four alkaloids, namely ephedrine (pseudoephedrine), 2,3,4-trimethyl-5-phenyloxazolidine, *trans*-1,2-dimethyl-3-phenylaziridine and 3,4-dimethyl-2,5-diphenyloxazolidine have been identified in the alkaloid extract of *Epdedra equisetina*. *In silico* anti-inflammatory activity of *Epdedra equisetina* alkaloids have been tested against inhibition of cyclooxygenases-1 enzyme (COX-1) (PDB code 3N8Z), cyclooxygenases-2 enzyme (COX-2) (PDB code 3LN1), and P38 mitogen-activated protein kinase (p38MAPK) (PDB code 1OZ1). Celecoxib has been used as a positive control. Molecular docking values of alkaloids in *Epdedra equisetina* range between –5.2 and –9.4 kcal/mol. The value for the reference drug (celecoxib) ranges between –6.6 and –12.2 kcal/mol. 3,4-Dimethyl-2,5-diphenyloxazolidine shows the highest molecular docking values of –7.0, –9.4, and –7.8 kcal/mol for COX-1, COX-2, and p38MAPK proteins, respectively. 3,4-Dimethyl-2, 5-diphenyloxazolidine can act as a natural alternative inhibitor of the COX-2 protein.

Keywords: *Ephedra equisetina* Bunge, Alkaloids, GC-MS analysis, Ephedrine, Anti-inflammatory activity

Ephedra species belongs to Ephedra L. genus, Ephedraceae Family. Nineteen Ephedra species are growing in the Flora of Tajikistan[1]. *Ephedra equisetina* Bunge is a small multi-branched shrub, perennial and up to 1.5 m tall. Perennial stems are covered with gray bark, and annual shoots are green in color. Its shoots are straight, smooth and thin. Leaves are opposite, 1-2 mm long[1]. In Tajikistan, the largest reserves of Ephedra plant are concentrated in Zarafshon mountains[2]. In the erstwhile Soviet Union, Ephedra plants were collected from Tajikistan for import[3].

Ephedra is one of the oldest medicinal plants. In folk medicine of Central Asia, its branches were used for colds, joint pain, rheumatism, malaria, stomach and liver diseases[2]. In modern medicine, broad pharmacological activities of Ephedra plant such as antiinflammatory, antioxidant, antiallergic, antiviral, diuretic and blood pressure regulatory activities have been documented[4]. Ephedra plants are rich in biologically active compounds, especially in alkaloids. More than 20 alkaloids were isolated from Ephedra plants[5]. However, the main bioactive alkaloids of the Ephedra plants ephedrine and pseudoephedrine. Quantity of these two alkaloids are consider for the quality test of Ephedra plants for it used in pharmaceutical industry[4]. In this relation, *Ephedra equisetina* Bunge is an important species due to the significant quantities alkaloids (up to 3.1%)[1,3].

The Ephedra plants have been used for their anti-inflammatory properties. It down-regulated upstream signaling pathways of pro-inflammatory mediators and cytokines in lipopolysaccharide[6]. The cyclooxygenase enzyme has two isoforms (COX-1 and COX-2) that are involved in different inflammatory processes[7]. Its inhibitors have been found to be effective in suppressing

inflammatory processes[8] p38 mitogen-activated protein kinases (p38MAPK) may regulate COX-2 expression[9]. In the present study, it is important to describe the molecular docking study of *Ephedra equisetina* alkaloids with COX-1, COX-2 and p38MAPK enzymes.

The present research deals with GC-MS analysis of the alkaloid extract of *Epdedra equisetina* growing wild in Tajikistan and *in silico* anti-inflammatory activity screening of its alkaloids.

Experimental Section

Reagents and Methods

All chemicals were purchased from Fluka, Sigma and Merk. Silica gel (Yantai Jiangyou Silica gel Development Co. Ltd., China; 200–300 mesh) was used for column chromatography. TLC (thin-layer chromatography) was used with HSGF254 plates (Yantai Jiangyou Silica gel Development Co.LTD, China). UV light (254 nm) and iodide vapors were used for visualizing spots on the TLC plates.

Plant Material

The aerial parts of wild *Ephedra equisetina* Bunge were collected on May 17, 2023, from Dehmanora village in the Kuhistoni Maschoh region of the Republic of Tajikistan at a height of 2800 meters above sea level. The authenticated voucher specimens of the plant (accession no. ICTNAS 2023-2) were deposited at the Laboratory of Heterocyclic Compounds of the V.I. Nikitin Chemistry Institute of the National Academy of Sciences of Tajikistan.

Extraction of the alkaloid extract of *Ephedra equisetina*

100 grams of dry powder of *Ephedra equisetina* plant mixed with 100 mL of water and 50 mL of 8 N hydrochloric acid solution and it kept for 5 hours. 350 mL of 95% ethanol was poured into the mixture, heated to boiling temperature and kept overnight. The next day, it filtered warm and washed with alcohol twice. Potassium carbonate and chloroform were added to the obtained filtrate until *p*H 9-10. The chloroform extract was filtered and dried using a vacuum rotor to obtain the alkaloid extract of *Ephedra equisetina*. The extraction yield was equal to 1.57%. The method of separation of the alkaloid extract is described in detail in[10].

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

The alkaloid extract of *Ephedra equisetina* was subjected to GC-MS analysis using an Agilent 6890 GC with Agilent 5973 MSD and HP-5ms (30 m × 0.25 mm × 0.25 μm) capillary column. The carrier gas was helium with a flow rate of 1.5 mL / min with split mode. The operating temperature conditions were initial temperature 60°C for 2 min isothermal followed by linear temperature increase up to 320°C at a rate of 3°C / min, and then for 10 min at isothermal mode at 320°C. Detector and injector temperatures were 320°C and 310°C, respectively. Identification of the essential oil components was based on retention indices (RI) and mass spectral fragmentation patterns with those reported in the literature[11,12] and NIST database.

Isolation of ephedrine (pseudoephedrine)

The alkaloid extract of *Ephedra equisetina* (3.2 g) was separated with column chromatography using a silica gel column (30 mm × 500 mm). Gradient elution was carried out with a chloroform: ethanol (4:1, 0:1, v/v) solvent system. TLC was used for testing separation.

Ephedrine (pseudoephedrine) quantitative test

Quantitative test of ephedrine (pseudoephedrine) was performed with Chen-Kao reaction[13].

In silico anti-inflammatory activity

We have used the mcule.com online platform to investigate ligand-target interactions. As ligands, we have used ephedrine, pseudoephedrine, 2,3,4-trimethyl-5-phenyloxazolidine, 1,2-dimethyl-3-phenylaziridine, and 3,4-dimethyl-2,5-diphenyloxazolidine, and as targets, we have applied cyclooxygenases-1 enzyme (COX-1) (PDB code 3N8Z), cyclooxygenases-2 enzyme (COX-2) (PDB code 3LN1), and P38 mitogen-activated protein kinase (p38MAPK) (PDB code 1OZ1). The celecoxib drug was used as a positive control. Binding site centers were for 3N8Z protein X 32.302; Y 44.1082; Z -32.0881, for 3LN1 protein X 18.6395; Y -52.3646; Z 54.125 and for 1OZ1 protein X 19.8049; Y 13.0568; Z 32.6076. Discovery Studio Visualizer (v.21.1.0.20298) was used for visualizing protein-ligand interactions[14].

Calculation of physicochemical properties

The physicochemical properties (molecular mass, partition coefficient, H-bond acceptors, H-bond donors, rotatable bonds, PSA (polar surface area), refractivity, atom number, ring number, heavy atoms, hydrogen atoms, heteroatoms, N/O atoms, and chiral centers) of the investigated alkaloids and positive control were calculated by the property calculator on the mcule.com online platform.

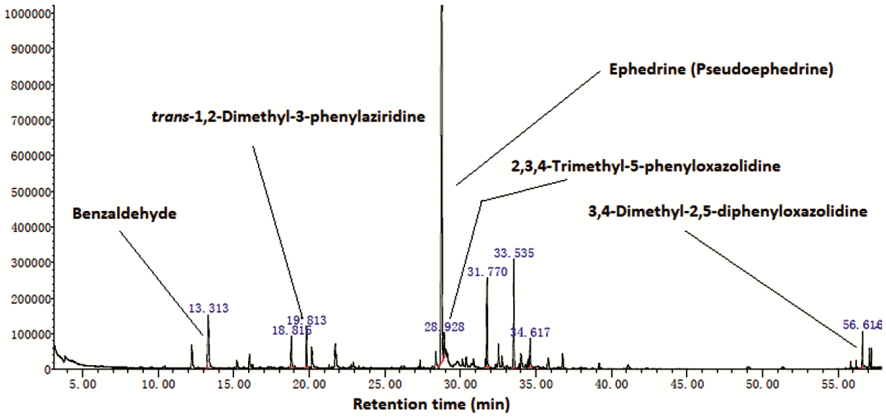

Fig. 1 — GC-MS profile of an alkaloid extract of *Epdedra equisetina* Bunge

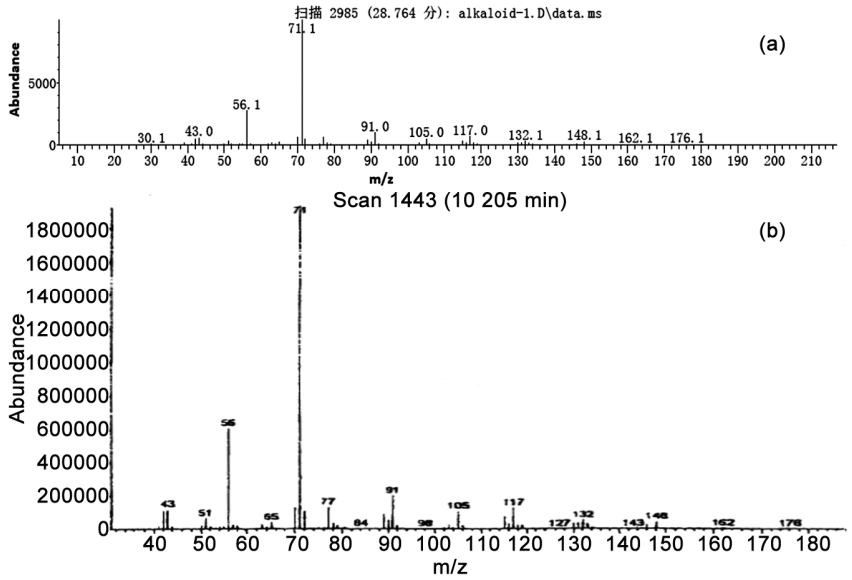

Fig. 2 — (A) Mass fragmentation (*m/z*) of main component of an alkaloid extract of *Epdedra equisetina* Bunge (RT 28.764 min), (B) Mass fragmentation (*m/z*) of ephedrine from PMW-TOX2.L database[11]

Toxicity test

The toxicity of the investigated alkaloids and the positive control were checked by the toxicity checker on the mcule.com online platform.

Results and Discussion

The aerial parts of wild *Epdedra equisetina* Bunge was collected from Maschohi Kuhi region of Tajikistan. The alkaloid extract of plant was obtained by acid-base extraction and analyzed by gas chromatography-mass spectrometry (GC-MS). GC-MS profile of an alkaloid extract of *Epdedra equisetina* Bunge is presented in Fig. 1.

Retention time of main component has 28.764 min. with mass fragmentation (*m/z*) 71.1 (100%); 56.1 (27.26%); 91.1 (9.91%); 117.0 (6.73%) and 77.0 (6.17%) (Fig. 2 (A)) that corresponds with ephedrine mass fragmentation (*m/z*) of represented in the PMW-TOX2.L mass spectral database (Fig. 2 (B)).

According to Tseng and co-authors ephedrine mass fragmentation can occurs by the scheme represented in Fig. 3. It can forms to main ions $[M\text{-}OH\text{-}C_6H_5]^+$ and $[M\text{-}OH\text{-}C_6H_5\text{-}CH_3]^+$ with *m/z* 71 and *m/z* 56, respectively[11].

Pursuant to our results, ephedrine and pseudo-ooephedrine were the dominant alkaloids, they consist 71.78% of the alkaloid content in *Epdedra equisetina.* 2,3,4-Trimethyl-5-phenyloxazolidine (1.71%), *trans*-1,2-dimethyl-3-phenylaziridine (2.64%) and 3,4-dimethyl-2,5-diphenyloxazolidine (1.85%) were identified in the alkaloid extract of *Epdedra equisetina.* The chemical composition of alkaloid contents of the wild *Epdedra equisetina* is represented in Table 1.

Among identified components, ephedrine and pseudo-oephedrine are amine containing alkaloids; 2, 3, 4-trimethyl-5-phenyloxazolidine and 3, 4-dimethyl-2, 5-diphenyloxazolidine are oxazolidine derivatives. The chemical structure of identified components is show in Fig. 4.

The alkaloid extract (3.2 g) of *Ephedra equisetina* was separated into 8 fractions. Two fractions were positive in Chen-Kao test reaction with weight 0.28 g. Yield of ephedrine (pseudoephedrine) was 8.75%.

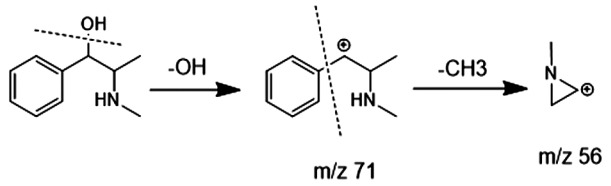

Fig. 3 — Mass fragmentation of ephedrine[11]

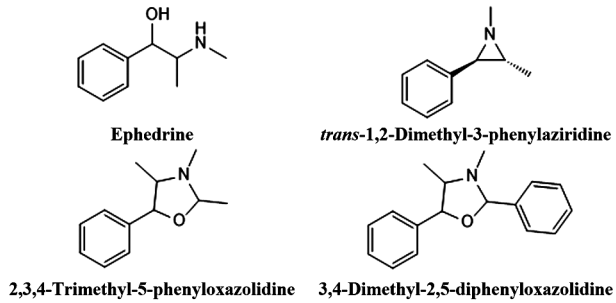

Fig. 4 — Chemical structure of the components of the alkaloid extract of *Ephedra equisetina* Bunge

According to literature, the highest total alkaloid content exists in *Ephedra equisetina* (2.5%) and of ephedrine content in the extract of different *Ephedra* species varies from 55% to 83% (Ref. 15). 2,3,4-Trimethyl-5-phenyloxazolidine and 3,4-dimethyl-2,5-diphenyloxazolidine are cyclized derivatives of ephedrine and pseudo- ephedrine that previously found in three *Ephedra* species (*E. sinica, E. intermedia* and *E. equisetina)* extracts[16,17]. Alkaloid 1,2-dimethyl-3-phenylaziridine was also found from aerial part of *Ephedra alata*[18].

Molecular docking values for five alkaloids of *Epdedra equisetina* were ranged between -5.2 and -9.4 kcal/mol (Table 2). The value for reference drug (celecoxib) was ranged between -6.6 and -12.2 kcal/mol. 3,4-Dimethyl-2,5-diphenyloxazolidine shows the highest molecular docking values -7.0; -9.4; and -7.8 kcal/mol for COX-1 (3N8Z); COX-2 (3LN1); and p38MAPK (1OZ1) proteins, respectively. Molecular docking values for positive control (Celecoxib) were -6.6; -12.2; and -8.4 kcal/mol for COX-1 (3N8Z); COX-2 (3LN1); and p38MAPK (1OZ1) proteins, respectively.

The mechanism of interaction of 3, 4-dimethyl-2, 5-diphenyloxazolidine and celecoxib with amino acids of the COX-2 (3LN1) protein is represented in Fig. 5. As can be seen from Fig. 5, the interaction of 3, 4-dimethyl-2, 5-diphenyloxazolidine and celecoxib with amino acids of the COX-2 (3LN1) protein has

Table 1 — Chemical composition of the alkaloid extract of *Epdedra equisetina* Bunge

RT	RI	Compd	Mass fragmentation (*m/z*)	Composition (%)
13.315	1142	Benzaldehyde	106.0 (100%); 105.0 (97.2%); 77.0 (84.23%); 51.0 (29.19%); 50.0 (17.27%); 78.0 (10.22%); 52.0 (9.15%); 39.0 (5.89%)	6.05
18.811	1268	Benzoic acid, methyl ester	105.0 (100%); 77.0 (52.94%); 136.0 (35.97%); 51.0 (16.58%); 106.0 (8.53%)	2.20
19.809	1290	*trans*-1,2-Dimethyl-3-phenylaziridine	146.1 (100%); 105.0 (36.19%); 132.0 (20.8%); 91.0 (14.15%); 147.0 (14.02%); 42.0 (14.01%)	2.64
28.764	1499	Ephedrine (Pseudoephedrine)	71.1 (100%); 56.1 (27.26%); 91.1 (9.91%); 117.0 (6.73%); 77.0 (6.17%)	71.78
28.927	1502	2,3,4-Trimethyl-5-phenyloxazolidine	85.1 (100%); 148.0 (52.35%); 70.0 (33.8%); 71.1 (22.87%); 90.0 (20.29%)	1.71
31.766	1569	Citric acid, methyl ester	143. 0 (100%); 101.0 (54.13%); 59.0 (13.75%); 175.0 (13.3%); 43.0 (7.47%)	5.26
33.538	1616	Citric acid, methyl ester	143.0 (100%); 101.0 (43.83%); 175.0 (14.87%); 157.0 (9.89%); 59.0 (9.31%)	6.36
34.613	1644	Isobutyl 3-(2-methoxyethyl) heptyl ester	157.0 (100%); 129.0 (69.29%); 101.0 (63.15%); 55.0 (34.8%); 83.0 (34.60%); 115.0 (25.3%); 143.0 (24.5%); 171.0 (10.8%)	2.15
56.617	2304	3,4-Dimethyl-2,5-diphenyloxazolidine	146.1 (100%); 147.1 (92.28%); 105.0 (27.24%); 148.05 (26.29%); 132.05 (24.46%); 91.0 (20%); 77.0 (20%)	1.85
		Total identified		100.0

Table 2 — Docking data of *Epdedra equisetina* alkaloids with COX-1, COX-2 and p38MAPK enzymes

Compd	COX-1 (3N8Z)	COX-2 (3LN1)	p38MAPK (1OZ1)
	Docking value (kcal/mol)		
Ephedrine	–6.1	–6.3	–5.2
Pseudoephedrine	–6.1	–6.3	–5.2
2,3,4-Trimethyl-5-phenyloxazolidine	–5.7	–7.5	–6.0
1.2-Dimethyl-3-phenylaziridine	–6.2	–6.4	–5.3
3,4-Dimethyl-2,5-diphenyloxazolidine	–7.0	–9.4	–7.8
Celecoxib (Positive control)	–6.6	–12.2	–8.4

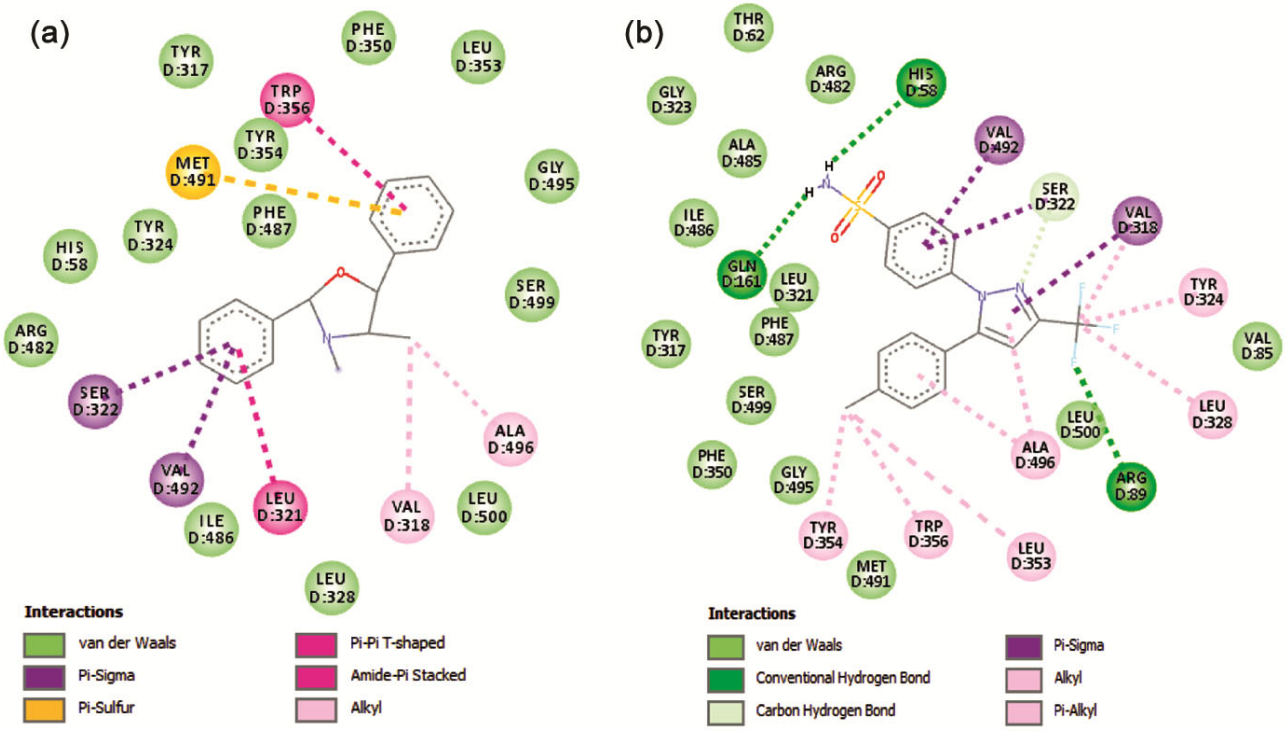

Fig. 5 — Snapshot represents the interaction between (**A**) 3,4-Dimethyl-2,5-diphenyloxazolidine, (**B**) Celecoxib with COX-2 (PDB ID 3LN1) protein

many similarities. Both interact with leucine (LEU D: 321, LEU D: 328, LEU D: 350, LEU D: 500), tyrosine (TYR D: 317, TYR D: 324, TYR D: 354), phenylalanine (PHE D: 350, PHE D: 478), serine (SER D: 322, SER D: 499), valine (VAL D: 318, VAL D: 492), tryptophan (TRP 356), methionine (MET D: 491), glycine (GLY D: 495), histidine (HIS D:58), arginine (ARG D: 482), alanine (ALA D: 496) amino acid residues that indicate the same mechanism of action. Our results are in agreement with previous published data that oxazolidine derivatives have potential anti-inflammatory activity[19-21].

Physicochemical properties such as molecular mass, partition coefficient, H-bond acceptors, H-bond donors, rotatable bonds, PSA (polar surface area), refractivity, atom number, ring number, heavy atoms, hydrogen atoms, heteroatoms, N/O atoms, and chiral centers of investigated compounds are demonstrated in Table 3. There may be a correlation between docking values (*in silico* anti-inflammatory activity) and molecular mass, partition coefficient, and heavy atoms of the Ephedra alkaloids.

The evaluation of the toxicity of the investigated alkaloids was performed by a toxicity checker. All alkaloids from *Epdedra equisetina* pass toxicity checks except 1, 2-dimethyl-3-phenylaziridine, which has a potential toxic substructure as shown in Fig. 6.

Table 3 — Physicochemical properties and toxicity checks of investigated compounds

Properties	Ephedrine	Pseudo-ephedrine	2,3,4-Trimethyl-5-phenyl oxazolidine	1,2-Dimethyl-3-phenyl aziridine	3,4-Dimethyl-2,5-diphenyl oxazolidine	Celeco-xib
Mass	165.231	165.231	191.269	147.216	253.338	381.373
logP	1.7188	1.7188	2.3621	1.9995	3.7149	5.2950
H-bond acceptors	2	2	2	1	2	8
H-bond donors	2	2	0	0	0	1
Rotatable bonds	3	3	1	1	2	4
PSA	32.26	32.26	12.47	3.01	12.47	86.36
Refractivity	49.7925	49.7925	61.2250	50.5260	80.9050	89.9624
Atoms	27	27	31	24	38	40
Rings	1	1	2	2	3	3
Heavy atoms	12	12	14	11	19	26
Hydrogen atoms	15	15	17	13	19	14
Heteroatoms	2	2	2	1	2	9
N/O atoms	2	2	2	1	2	5
Chiral centers	2	2	3	3	3	0
R/S chiral centers	2	2	0	0	0	0
Toxicity check	Non toxic	Non toxic	Non toxic	Toxic	Non toxic	Non toxic

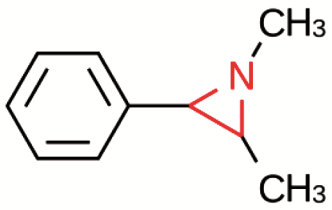

Fig. 6 — Potential toxic substructure (red colour) of 1,2-dimethyl-3-phenylaziridine

Conclusion

Epdedra equisetina is a medicinal plant used for a long time in Tajik folk medicine. The alkaloid extract of the plant was obtained by acid-base extraction and analyzed by gas chromatography-mass spectrometry (GC-MS) and column chromatography (CC). According to the GC-MS and CC data, ephedrine was the main component in *Epdedra equisetina* alkaloid extract; its contents determined 71.78% and 8.75%, respectively. Results of molecular docking indicate that 3,4-dimethyl-2,5-diphenyloxazolidine can be a natural alternative inhibitor of the COX-2 protein.

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